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(54) Use of histone deacetylase inhibitors for treating fribosis or cirrhosis

(57) The invention relates to a pharmaceutical composition for the treatment of fibrosis, in particular liver fibrosis and cirrhosis.

According to the invention it was surprisingly found that submicromolar concentrations of a histone deacetylase inhibitor and in particular of trichostatin A strongly inhibit three main features of myofibroblastic differentiation of cultured hepatic stellate cells: (1) synthesis of collagens type I and III, the predominant collagens in fibrosis of liver and other organs; (2) cellular proliferation; and (3) expression of smooth muscle α-actin, a marker for differentiated myofibroblasts. These results indicate that trichostatin A is a potent antifibrogenic agent, totally different from any other therapeutic compounds previously described.

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Description

The invention relates to a pharmaceutical composition for the treatment of fibrosis, in particular liver fibrosis and cirrhosis.

Liver fibrosis is a pathological condition characterized by excessive deposition of connective tissue proteins. Rather than a single disease entity, it is a condition resulting from various diseases, including viral hepatitis, alcoholic liver disease, schistosomiasis, etc. Regardless of the initial cause of the disease, the liver parenchyma is progressively replaced by connective tissue, resulting in deterioration of liver functions.

To date, no truly effective therapeutic drug exist for the treatment of fibrotic diseases in particular of the liver.

Hepatic stellate cells are the major connective tissue producing cells in both normal and fibrotic livers. In the normal situation, stellate cells serve as vitamin A storage site. These cells are quiescent, show little proliferative activity, and express a limited spectrum of connective tissue proteins. In injured or fibrotic livers, however, stellate cells lose their fat-droplets and change their phenotype into myofibroblast-like cells. These myofibroblast-like cells are "activated" cells, show high proliferative activity, and produce large amounts of collagens and other extracellular matrix proteins. Accordingly, this cell type is the logical target for therapeutic intervention. Compounds with antifibrotic effect on stellate cells will be a promising candidate molecule for the treatment of liver fibrosis and cirrhosis.

According to the invention it was surprisingly found that submicromolar concentrations of a histone deacetylase inhibitor and in particular of trichostatin A strongly inhibit three main features of myofibroblastic differentiation of cultured hepatic stellate cells: (1) synthesis of collagens type I and III, the predominant collagens in fibrosis of liver and other organs; (2) cellular proliferation; and (3) expression of smooth muscle α-actin, a marker for differentiated myofibroblasts. These results indicate that trichostatin A is a potent antifibrogenic agent, totally different from any other therapeutic compounds previously described.

The present invention is directed to the use of a histone deacetylase inhibitor or a pharmaceutical acceptable salt thereof for the preparation of a pharmaceutical composition for the treatment of fibrosis, in particular liver fibrosis and/or cirrhosis.

Three classes of compounds are currently known as inhibitors of histone deacetylase. Sodium butyrate is a natural 4 carbon fatty acid that inhibits histone deacetylase in a non-competitive manner, and requires millimolar concentrations for its biologic activities. Trichostatin A and trapoxin are specific inhibitors of histone deacetylase, and are much more potent than sodium butyrate, being effective in the submicromolar range.

The description in this application is in particular directed to trichostatin A as a non-limiting example and is never intended to limit the scope of the invention.

Trichostatin A or derivatives thereof are disclosed to be useful as an antifibrotic agent for the treatment fibrosis. Pharmaceutical formulations and use of compounds of Trichostatin A are also disclosed.

Trichostatin A is an antifungal agent originally isolated from Streptomyces hygroscopicus by Tsuji et al. (J. Antibiot 29:1-6, 1976). Trichostatin A is also useful as an anticancer (Cancer Res 47:3688-3691, 1987) and an antiprotozoal agent (J. Antibiot 41:461-468, 1988). In the course of experiments we discovered that Trichostatin A has a strong antifibrotic effect on hepatic stellate cells which are the major connective tissue producing cells in the liver.

Trichostatin A can be brought in the form of pharmaceutically acceptable salts. As such pharmaceutically acceptable salts may be used so long as they do not adversely affect the desired pharmacological effects of the compounds. The selection and the production can be made by those skilled in the art. For instance, as a pharmaceutically acceptable salt, and alkali metal salt such as sodium salt or a potassium salt, an alkaline earth metal salt such as calcium salt or a magnesium salt, a salt with an organic base such as an ammonium salt, or a salt with an organic base such as a triethylamine salt or an ethanolamine salt, may be used.

Subjects to be treated by the present invention include both humans and animals.

The antifibrotic agent of the present invention may be administered orally or non-orally. In the case of oral administration, it may be administered in the form of soft and hard capsules, tablets, granules, powders, solutions, suspensions or the like. In the case of non-oral administration, they may be administered in the form of injection solution, drip infusion formulations, suppositories whereby continuous membrane absorption can be maintained in the form of solid, viscous liquid, or suspension. The selection of the method for preparation of these formulations and the vehicles, disintegrators or suspending agents, can be readily made by those skilled in the art. The antifibrotic agent of the present invention may contain a further substance having antifibrotic activities, in addition to Trichostatin A or its pharmaceutically acceptable salts.

The amount of the active ingredients in the composition of the present invention may vary depending on the formulation, but is usually from 0.1 to 50 % by weight irrespective of the manner of administration. The dose is determined taking into consideration the age, sex, and symptom of the disease of the subject, the desired therapeutic effect, the period for administration, etc. However, preferably a daily dose of the active ingredient is from 0.05 to 100 mg for an adult.

The following examples are provided to illustrate the present invention, and should not be construed as limiting

thereof.

Trichostatin A was prepared from the culture broth of Streptmyces platensis No.145. Sodium butyrate was purchased from Sigma (St. Louis, MO, USA). Stock solutions of Trichostatin A were prepared in ethanol (2 mg/ml), stored at -20°C, and diluted as required for each experiment. The final concentration of ethanol in the medium was 0.0016%. Stock solutions of sodium butyrate (100 mmol/L) were prepared in distilled water, and diluted as required.

Example 1

Effect of Trichostatin A and sodium butyrate on the synthesis of collagens type I and III, and smooth muscle α-actin by hepatic stellate cells

The antifibrotic activity of Trichostatin A and sodium butyrate were tested using cultures of hepatic stellate cells.

Stellate cells were isolated from adult Wistar rats (400-550 g) by enzymatic digestion of the liver with collagenase/pronase/DNAase followed by density gradient centrifugation on Nicodenz (Nycomed, Oslo, Norway).

After isolation cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, and cultured at 37°C in a humidified atmosphere with 5 % CO_2 and 95 % air.

At day 3 cells were exposed to Trichostatin A (1-100 nmol/L) for 24 h. During the subsequent 24 h cells were metabolically labeled with 25 µCi/ml of Trans ^{35}S-label (specific activity of ^{35}S-methionine > 1,000 Ci/mmol, ICN Biomedicals, Costa Mesa CA) while exposure to the compounds was continued. After labelling medium was collected, and subjected to immunoprecipitation using antibodies against collagens type I and III and smooth muscle α-actin. The precipitates were fractionated by SDS-PAGE and radioactivity of specific bands were measured by the PhosphorImaging technology. For the effect at mRNA level, cells were exposed to 100 nmol/L Trichostatin A for 24 h. RNA was then extracted and analyzed by Northern hybridization analysis.

Table 1 and 2 show the results which were expressed as percentage value relative to the control culture, respectively for Trichostatin A and sodium butyrate. Note the dose-dependent suppression of collagens type I and III synthesis by Trichostatin A. Also, note the strong suppression of smooth muscle α-actin, an activation marker of stellate cells.

Table 1

Results for Trichostatin A			
	100 nM	10 nM	1 nM
collagen I	37.9±5.6	68.9±4.7	91.7±9.5
collagen III	30.1±9.6	73.2±20.9	71.9±21.0
SM α-actin	15.5±7.4	54.4±5.3	87.6±0.3

Sodium butyrate suppressed smooth muscle α-actin less effectively, with 50% reduction at a concentration of 1 mmol/L. Inhibition of collagen type III and smooth muscle α-actin synthesis indicated that Trichostatin A was more potent than butyrate by 5 orders of magnitude.

Table 2

Results for sodium butyrate			
	10^{-3} M	10^{-4} M	10^{-5} M
collagen I	107.8±9.8	127.9±16.2	92.8±11.7
collagen III	67.9±19.1	90.6±42.2	109.4±31.1
SM α-actin	50.0±19.9	91.6±23.4	97.9±24.4

Example 2

Effect of Trichostatin A on the gene expression of collagens type I and III, and smooth muscle α-actin by hepatic stellate cells

Hepatic stellate cells were isolated and cultured as described in Example 1. Cells were exposed to 100 nmol/L Trichostatin A for 24 h. RNA was then extracted and analyzed by Northern hybridization analysis.

At day 3 cells were exposed to 100 nmol/L Trichostatin A or 1 mmol/L sodium butyrate for 24 h. Total RNA was extracted by the method of Chomczynski and Sacchi. Northern hybridization was performed using P-labeled cDNA probes for rat procollagen α_1(I) (1.6 kb Pst I fragment), rat procollagen α_1(III) (0.5 kb Hind III/Eco-RI fragment), and GAPDH (0.5 kb XbaI/HindIII fragment). For smooth muscle α-actin, a cRNA probe corresponding to the 5'-untranslated region of mouse smooth muscle α-actin mRNA was used as described previously. The results were quantitated by PhosphorImager and corrected for GAPDH.

The results at the mRNA level are shown in Table 3. Collagen type III and smooth muscle α-actin mRNA levels were suppressed to a similar extent as at the protein level. On the other hand, only modest tendency for suppression was observed for collagen type I, suggesting that the suppression of collagen type I was mainly post-translational.

Table 3

	100 nM
collagen I	79.3±13.5
collagen III	39.0±13.5
SM α-actin	20.6±15.4

Example 3

Effect of Trichostatin A and sodium butyrate on the cell proliferation of hepatic stellate cells

Finally, the applicant examined the effects of Trichostatin A and sodium butyrate on proliferation of stellate cells, since high proliferative activity is one of the major features of myofibroblastic differentiation.

Cells were cultured in triplicate or quadruplicate in 24 well plates (Costar). Cells at day 2 were exposed to 0.01-1 mmol/L sodium butyrate or 1-100 nmol/L Trichostatin A for the 4 subsequent days. Culture medium and test compounds were replaced every day. At day 6 cells were trypsinized and counted in a hemocytometer.

Trichostatin A showed at 100 nmol/L a strong suppressive effect on proliferation. Table 4 summarizes these cell-count results.

Table 4

Cell-count results			
control	10^{-7} M Trichostatin A	10^{-8} M Trichostatin A	10^{-9} M Trichostatin A
23.7±1.9	16.2±1.0	22.0±3.0	22.9±1.0
control	10^{-3} M Butyrate	10^{-4} M Butyrate	10^{-5} M Butyrate
23.9±2.0	20.4±0.2	22.2±0.7	21.1±2.0

Finally cells were cultured in triplicate of quadruplicate in 24 well plates. At day 4 cells were exposed tot 0.01-1 mmol/L sodium butyrate or 1-100 nmol/L Trichostatin A for 24 h. Subsequently, medium was changed and cells were further incubated for 20 h with the same concentrations of sodium butyrate or Trichostatin A in the presence of 10 µCi/ml [^{3}H]-thymidine (specific activity 25 Ci/mmol, 10 µCi/ml). Radioactivity incorporated into the 2% perchloric acid/95% ethanol/insoluble fraction was measured by scintillation counting. Parallel cultures incubated with [^{3}H]-thymidine in the presence of 10 mmol/L hydroxyurea provided the baseline value, which was subtracted from each measurement. Final data were normalized for cell number which was determined by trypsinization of parallel wells. Table 5 shows the results expressed in cpm/cell.

Table 5

[^{3}H]-thymidine incorporation			
control	10^{-7} M Trichostatin A	10^{-8} M Trichostatin A	10^{-9} M Trichostatin A
10.0±0.8	1.3±0	9.9±0.4	9.6±0.4
control	10^{-3} M butyrate	10^{-4} M butyrate	10^{-5} M butyrate
9.9±0.4	6.2±0.2	9.5±0.3	9.5±0.2

Similar results were obtained with trapoxin, which is another histone deacetylase inhibitor. The results in this application indicate that histone deacetylase inhibitors provide a novel therapeutic potential in the treatment of fibro proliferative diseases.

The invention further relates to a method for the treatment of humans or animals afflicted with fibrosis and in particular liver fibrosis or cirrhosis, comprising administering to said subject an effective amount of a histone deacetylase inhibitor in particular Trichostatin A or a pharmaceutical acceptable salt thereof and optionally a suitable excipient.

Claims

1. Use of a histone deacetylase inhibitor for the preparation of a pharmaceutical composition for the treatment of fibrosis, in particular liver fibrosis and/or cirrhosis.

2. Use according to claim 1, wherein the histone deacetylase inhibitor is trichostatin A or a pharmaceutical acceptable salt thereof.

3. Use according to claim 1, wherein the histone deacetylase inhibitor is trapoxin or a pharmaceutical acceptable salt thereof.

4. Use according to claim 1, wherein the histone deacetylase inhibitor is sodium butyrate.

5. Use according to claim 1-4, wherein the amount of active ingredient varies from 0.1 to 50% by weight.

6. Use according to claim 5, wherein the daily dose of the active ingredient for an adult is from 0.05 to 100 mg.

7. Use according to claim 1-6, wherein the pharmaceutical salt is an alkali metal salt, such as sodium salt or a potassium salt, an alkaline earth metal salt such as calcium salt or a magnesium salt, a salt with an organic base such as an ammonium salt, or a salt with an organic base such as a triethylamine salt or an ethanolamine salt.

8. Method for treating fibrosis comprising administering to humans or animals in need of an anti-fibrotic treatment as anti-fibrotic agent a therapeutically effective amount of a pharmaceutical composition according to claim 1-7.

9. Method for treating fibrosis according to claim 8, wherein the pharmaceutical composition is administered non-orally, in particular in the form of a injection solution, drip infusion or suppositories.

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EUROPEAN SEARCH REPORT

Application Number
EP 96 20 2460

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
Y	EXP. CELL RES., vol. 177, no. 1, 1988, pages 122-131, XP000617221 M. YOSHIDA: "Reversible arrest of proliferation of rat 3Y1 fibroblasts in both the G1 and G2 phases by trichostatin A" * the whole document * ---	1-9	A61K31/165 A61K31/19 A61K38/12
Y	J. BIOL. CHEM., vol. 265, no. 28, 1990, pages 17174-17179, XP000616087 M. YOSHIDA: "Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A" * the whole document * ---	1-9	
Y	J. BIOL. CHEM., vol. 268, no. 30, 1993, pages 22429-22435, XP000616088 M. KIJIMA: "Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase" * the whole document * ---	1-9	TECHNICAL FIELDS SEARCHED (Int.Cl.6) A61K
Y	BIOCHEM. PHARMACOL., vol. 37, no. 19, 1988, pages 3771-3776, XP000645000 A.M. GRESSNER: "Effect of n-butyrate on the synthesis of sulfated glycosaminoglycans and hyaluronate by rat liver fat-storing cells (Ito cells)" * the whole document * -----	1-4	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	27 February 1997	Orviz Diaz, P

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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